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ORIGINAL ARTICLE

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Multiplex PCR, amplicon size and hybridization efficiency on the NanoChip electronic microarray

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Abstract We tested the SNP typing protocol developed for the NanoChip electronic microarray by analyzing the four Y chromosome loci SRY1532, SRY8299, TAT, and 92R7. Amplicons of different lengths containing the same locus were purified and addressed to the NanoChip array and fluorescently labelled reporter probes were hybridized to the amplicons. We demonstrated that as little as 10–30 fmol of 50 bp DNA amplicons was sufficient to obtain strong and reproducible results. The hybridization to 50 bp amplicons was up to 10 times more efficient than the hybridization to 200 bp amplicons containing the same SNP. Hybridization to individual amplicons in multiplexes was less efficient suggesting that intramolecular and intermolecular interactions may block access to the target sequence on the NanoChip array. We observed a high risk of contamination with amplicons shorter than 60 bp and therefore, we recommend the use of 60–200 bp amplicons for SNP typing analysis on the NanoChip platform. In a comparative study, we typed the 5 Y chromosome loci M173, 92R7, P25, SRY1532, and M9 in 400 males using the NanoChip SNP typing protocol and the SNaPshot kit. Concording results were obtained for all samples demonstrating the accuracy of the NanoChip SNP typing protocol.

Keywords Electronic microarray · Hybridization · Amplicon size · SNP typing

Introduction

One of the most interesting new microarray technologies is the NanoChip electronic microarray [1, 2]. The electronic capability of this platform provides the potential for a high degree of automatization and the opportunity to electronically direct and concentrate sample material to specific sites within the array increases the sensitivity and the specificity of the assay in question. These advantages have been exploited and several very different applications have been reported, including cell separation [3, 4], on-chip strand displacement amplification [5, 6, 7], gene expression analysis [8], immunoassays [9], STR analysis [10], SNP analysis [11] and mutation screening [12].

In this work, we analyzed the sensitivity of the NanoChip SNP typing protocol. We analyzed the hybridization efficiency towards PCR amplicons of different lengths and we explored the possibility to detect SNPs in small PCR multiplexes. We decided to use Y chromosome SNPs for this work because minimal rules for SNP scoring can be applied when the possibility of heterozygotes is eliminated. This makes the Y chromosome SNPs ideal for testing the maximum sensitivity of the NanoChip SNP typing protocol.

In the present SNP typing protocol, biotin-labelled PCR products were electronically addressed to the array and bound to streptavidin embedded in the permeation layer above the electrodes. Two fluorescently labelled reporter oligonucleotides, one for each SNP allele, and one stabilizer oligonucleotide were hybridized to the biotin-labelled strand. The reporter oligonucleotides were short (8–14 bp) whereas the stabilizer oligonucleotide was relatively long (25–30 bp). The SNP was positioned at the 3'-end of the reporter and the 5'-end of the stabilizer oligonucleotide hybridized to the neighboring base. The close proximity of the matching reporter and the stabilizer oligonucleotide created a base-stacking effect between the two oligonucleotides that stabilized the binding of the matching reporter to the target [13, 14]. In contrast, the reporter carrying a mismatch in the 3'-end bound weakly to the target. After stringent washes performed on the microarray, only the matching reporter bound to the target and the fluorescent label on the matching reporter was excitated by laser light and detected by a CCD camera.

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Materials and methods

DNA preparation and quantification

A total of 400 unrelated males and 5 unrelated females donated blood samples. From samples collected on FTA paper (Whatman, Cambridge, UK), a 1.2 mm disk was isolated. The disk was washed twice with 200 µl ddH_2O for 15 min on a shaker, allowed to dry, and was subsequently used for PCR. From liquid blood samples, DNA was isolated from 200 µl of peripheral blood using the QIAamp DNA Blood Mini kit (Qiagen, Albertslund, Denmark). DNA was quantified in a LightCycler (Roche, Hvidovre, Denmark) using SYBR-green I (Bie & Berntsen, Rødovre, Denmark) as previously described [15]. Standard curves were made using a calf thymus DNA standard (InVitrogen, Taastrup, Denmark). The variation between double determinations was less than 25 %.

PCR conditions

Singleplex PCR amplification of TAT, SRY8299, SRY1532 and 92R7 was performed in 25 µl reactions containing 2 U AmpliTaq Gold polymerase (Applied Biosystems, Copenhagen, Denmark), 1× AmpliTaq Gold PCR buffer, 2 or 3 mM $MgCl_2$, 200 µM dNTP and 0.5 µM of each primer using the following programme: denaturation for 10 min at 95°C, 32 cycles of 94°C for 30 s, 56°C for 20 s and 72°C for 1 min, followed by 10 min at 72°C. A 4-plex PCR amplification of TAT, SRY8299, SRY1532 and 92R7 was performed in 25 µl reactions containing 2 U AmpliTaq Gold polymerase, 1× AmpliTaq Gold PCR buffer, 3 or 4 mM $MgCl_2$, 200 µM dNTP and 0.5 µM of each primer using the following programme: denaturation for 10 min at 95°C, 32 cycles of 94°C for 30 s, 52°C (short 4-plex) or 56°C (medium 4-plex) or 62°C (long 4-plex) for 20 s and 65°C for 1 min, followed by 10 min at 72°C. Multiplex PCR amplification of SRY1532, M9, P25, 92R7 and M173 was performed in 50 µl reactions containing 2.5 U AmpliTaq Gold polymerase, 1× AmpliTaq Gold PCR buffer, 8 mM $MgCl_2$, 400 µM dNTP and 0.01–0.42 µM of each primer using the following programme: denaturation for 5 min at 94°C, 33 cycles of 95°C for 30 s, 60°C for 30 s and 65°C for 30 s, followed by 7 min at 65°C [16]. All PCR amplifications were performed in a GeneAmp 9600 thermal cycler (Perkin Elmer, Ueberlingen, Germany) or in a MasterCycler gradient (Eppendorf, Hamburg, Germany). Oligonucleotides were purchased from TAG Copenhagen (Copenhagen, Denmark) or DNA Technology (Aarhus, Denmark).

Purification of PCR products

PCR products longer than 100 bp were purified using MultiScreen PCR plates in a MultiScreen separation system (Millipore, Copenhagen, Denmark) as recommended by the manufacturer and 100 µl ddH_2O was used for washing. Samples were typically recovered in 80 µl 50 mM histidine. PCR products shorter than 100 bp were purified by MinElute PCR purification spin columns (Qiagen, Albertslund, Denmark) as recommended by the manufacturer and DNA was eluted in 30 µl ddH_2O.

Gel electrophoresis

PCR products were separated in 11% polyacrylamide gels by electrophoresis in 1× TBE buffer and the gels were stained with 0.5 µg/ml ethidium bromide. A 10 bp DNA ladder (InVitrogen, Taastrup, Denmark) or *Hae*III digested ϕX-174-RF (Amersham Biosciences, Hillerød, Denmark) was used as marker. Photos were taken of the gels using the GDS8000 gel documentation system (UVP, Cambridge, UK) and the photos were analyzed by the LabWorks 4.0 software (UVP, Cambridge, UK).

Design of reporter and stabilizer oligonucleotides

The following guidelines were used:

1. The SNP was positioned at the 3'-end of the reporter
2. The theoretical Tm's of the reporters were between 31 and 42°C
3. The theoretical Tm of the two reporters did not differ by more than 2°C
4. Palindromic sequences longer than 4 nucleotides were avoided within the reporters in order to avoid primer-dimer formation
5. Low base-stacking energies (T:A, A:A, and T:T) were avoided whenever possible
6. The base-stacking energies were similar whenever possible
7. Sequences causing hairpin structures with strong binding forces ($\Delta G < -7$ kcal/mol) and palindromic sequences longer than 4 nucleotides were avoided in the stabilizer oligonucleotide
8. The theoretical Tm of the stabilizer was close to 60°C. The theoretical Tm was calculated by the OligoAnalyzer 3.0 software (http://www.idtdna.com) using the nearest neighbor algorithm.

Table 1 Primers for PCR of Y chromosome SNPs

Y chromosome SNP	Primers
TAT	Forward 5'Biotin-CTTGGGAAAAATACACTACGTC (279 bp)
	Forward 5'Biotin-CTCTGAGTGTAGACTTGTG (108 bp and 54 bp)
	Reverse GAAGGTGCCGTAAAAGTGTG (279 bp and 108 bp)
	Reverse GTGCTCTGAAATATTAAATTAAAAC (54 bp)
SRY1532	Forward 5'Biotin-TCCTTAGCAACCATTAATCTGG (165 bp)
	Forward 5'Biotin-GGCCTCTTGTATCTGAC (63 bp)
	Reverse ATAGCAAAAAATGACACAAGGCA (165 bp)
	Reverse CACCACATAGGTGAACC (63 bp)
SRY8299	Forward 5'Biotin-AGCACATTAGCTGGTATGAC (267 bp and 167 bp)
	Forward 5'Biotin-AGCCCTTCGAGAGGTCAAG (44 bp)
	Reverse CCGAGTAACTGGGATTACA (267 bp)
	Reverse TCTCTTCACCCTGTGATCCG (167 bp and 44 bp)
92R7	Forward 5'Biotin-CTCAGCCTCCCAAAGTTC (316 bp)
	Forward 5'Biotin-GTTAAATATGACCAGCAAAGAC (210 bp and 48 bp)
	Reverse CTTCAGTGATTTCTGGGTAGC (316 bp and 210 bp)
	Reverse CATGAACACAAAAGACGTAG (48 bp)

The sizes of the resulting PCR products are shown in parentheses.

Hybridization on the NanoChip electronic microarray

Biotinylated PCR products or 1–6 nM biotinylated control oligonucleotides (Table 1) were diluted in 50 mM histidine in a total volume of 60 µl or 100 µl. A volume of 100 µl was used if the loading protocol lasted longer than 3 h in order to account for evaporation during loading of the NanoChip cartridge (Nanogen, Helmond, Netherlands). DNA was electronically addressed to individual pads on the NanoChip array using the Nanogen Molecular Biology Workstation (Nanogen, Helmond, Netherlands) as recommended by the manufacturer. NCC-2004 NanoChip cartridges were used for all experiments. Loading times for samples and control oligonucleotides were 3 and 2 min, respectively. After loading, the NanoChip array was treated with 0.1 M NaOH for 3 min to remove the unlabelled strands of the PCR products. The NanoChip array was washed 3 times in 150 µl high salt buffer (500 mM NaCl, 50 mM NaH_2PO_4, pH 7.0). The last wash was left in the NanoChip array for 5 min to ensure that the permeation layer above the electrodes was saturated with high salt buffer: 1 µM of each fluorescently labelled reporter oligonucleotide and 250 nM stabilizer oligonucleotide (Table 2) were diluted in 150 µl high salt buffer and added to the NanoChip array. Hybridization was performed at room temperature for 5 min. After the hybridization, the NanoChip array was washed 3 times in 150 µl high salt buffer. The NanoChip array was loaded in the Nanogen Molecular Biology Workstation and washed with 150 µl low salt buffer (50 mM NaH_2PO_4, pH 7.0). The fluorescence signal was scanned at different temperatures using the laser and the detector on the Nanogen Molecular Biology Workstation [1, 2]. The NanoChip array was washed twice with low salt buffer at each temperature. If multiple DNA fragments were loaded in the same pad on the NanoChip array, the reporter oligonucleotides and stabilizer oligonucleotide were removed by treating the NanoChip array with 0.1 M NaOH for 3 min. Another SNP in the multiplex was subsequently analyzed by another round of washing and hybridization with a new set of reporters and stabilizer. The control oligonucleotides for each SNP were loaded in four different pads. Each control oligonucleotide was loaded alone in one pad and two pads were loaded with both control oligonucleotides. The pads with both control oligonucleotides were used to normalize the fluorescent signal. A pad loaded with 50 mM histidine was used for background subtraction. The images shown in Figs. 1, 2 and 3 are raw data without normalization. The data were

Table 2 Reporters, stabilizers, and control oligonucleotides

Y chromosome SNP	Nucleotide sequence
TAT	Reporter 5'CY5-AAATTAAAACAACA
	Reporter 5'CY3-AATTAAAACAACG
	Stabilizer TGAATTCACAAGTCTACACTCAGAG
	Control 5'Biotin-CTCTGAGTGTAGACTTGTGAATTCA**T**GTTGTTTTAATTTAATATTTCAGAGCAC
	Control 5'Biotin-CTCTGAGTGTAGACTTGTGAATTCA**C**GTTGTTTTAATTTAATATTTCAGAGCAC
SRY1532	Reporter 5'CY5-CTTGAAAATGTTAC
	Reporter 5'CY3-CTTGAAAATGTTAT
	Stabilizer ACTGTGTGAAAAAGTCAGATACAAGAGGCC
	Control 5'Biotin-AGTATCTGGCCTCTTGTATCTGACTTTTTCACACAGT**G**TAACATTTTCAAGGTTCAC
	Control 5'Biotin-AGTATCTGGCCTCTTGTATCTGACTTTTTCACACAGT**A**TAACATTTTCAAGGTTCAC
SRY8299	Reporter 5'CY5-GTGATCCGCTC
	Reporter 5'CY3-GTGATCCGCTT
	Stabilizer GCCTTGACCTCTCGAAGGGCTGG
	Control 5'Biotin-TGTAATCCCAGCCCTTCGAGAGGTCAAGGC**G**AGCGGATCACAGGG
	Control 5'Biotin-TGTAATCCCAGCCCTTCGAGAGGTCAAGGC**A**AGCGGATCACAGGG
92R7	Reporter 5'CY3-GACGTAGAAGC
	Reporter 5'CY5-AGACGTAGAAGT
	Stabilizer TTGTCTTTGCTGGTCATATTTAACAATGCTAATTTGGTTA
	Control 5'Biotin-GTTAAATATGACCAGCAAAGACAA**G**CTTCTACGTCTTTTGTGTTCATG
	Control 5'Biotin-GTTAAATATGACCAGCAAAGACAA**A**CTTCTACGTCTTTTGTGTTCATG
P25	Reporter 5'CY3-ACCTGCCTGC
	Reporter 5'CY5-AACCTGCCTGA
	Stabilizer AAATAGAATTGTGCTCGTATCTCGGTCCAT
	Control 5'Biotin-ATGATGGACCGAGATACGAGCACAATTCTATTT**G**CAGGCAGGTTTCA
	Control 5'Biotin-ATGATGGACCGAGATACGAGCACAATTCTATTT**T**CAGGCAGGTTTCA
M173	Reporter 5'CY5-AGATGACAAAGG
	Reporter 5'CY3-AGATGACAAAGT
	Stabilizer GTTCTAAATGCCCTTGAATTGTAAGAA
	Control 5'Biotin-TTTTCTTACAATTCAAGGGCATTTAGAAC**C**CTTTGTCATCTGTTAATA
	Control 5'Biotin-TTTTCTTACAATTCAAGGGCATTTAGAAC**A**CTTTGTCATCTGTTAATA
M9	Reporter 5'CY5-GATGGTTGAATG
	Reporter 5'CY3-GATGGTTGAATC
	Stabilizer CTCTTTATTTTTCTTTAATTTAGACATGTTCA
	Control 5'Biotin-GTAAGACATTGAACGTTTGAACATGTCTAAATTAAAGAAAAATAAAGAG**G**ATTCAACCATCTTAGG
	Control 5'Biotin-GTAAGACATTGAACGTTTGAACATGTCTAAATTAAAGAAAAATAAAGAG**C**ATTCAACCATCTTAGG

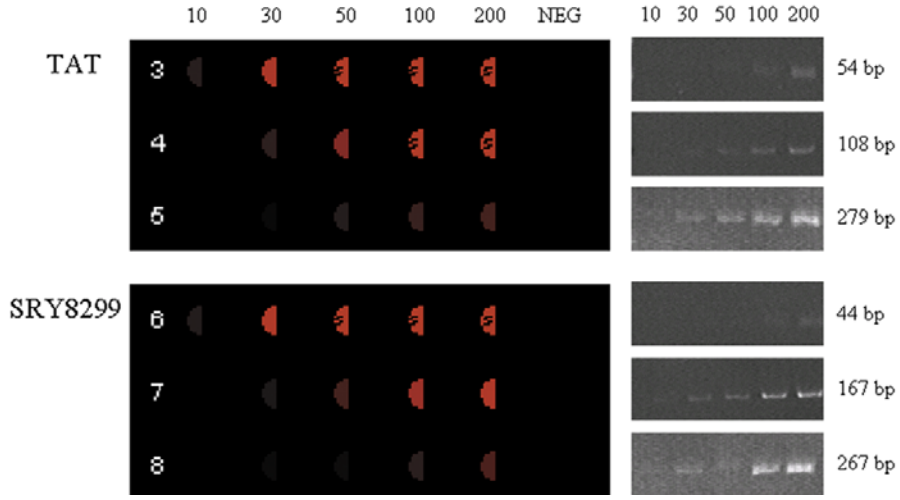

Fig. 1 Hybridization experiment with TAT and SRY8299 singleplex PCR products. A total of 10 µl 1, 3, 5, 10, and 20 fmol/µl dilutions and a negative PCR control were loaded in polyacrylamide gels and addressed to the NanoChip array. The PCR products were loaded in the following order on the NanoChip: *row 3* 54 bp TAT fragments, *row 4* 108 bp TAT fragments, *row 5* 279 bp TAT fragments, *row 6* 44 bp SRY8299 fragments, *row 7* 167 bp SRY8299 fragments, *row 8* 267 bp SRY8299 fragments. The corresponding gel images are shown on the right. Hybridization was performed at 33°C and at 44°C for TAT and SRY8299, respectively. *s* inside the coloured half circle indicates that the fluorescent signal was saturated

collected and analyzed with the nLAB 1.09.09c software (Nanogen, San Diego, USA).

Results

Hybridization efficiency depends on the size of the amplicon

We selected four known Y chromosome SNPs, SRY1532, SRY8299, TAT and 92R7 [17] and we designed PCR primers that would amplify DNA fragments of different lengths for each SNP (Table 1). The size of the PCR products ranged from 44 bp to 316 bp. During the initial experiments, we learned that fragments shorter than 100 bp were lost completely during purification if we used the Multiscreen Purification System. Therefore, we decided to use the MinElute PCR purification kit for purification of fragments shorter than 100 bp. Recoveries for both purification methods were approximately 80%. However, PCR products purified by the MinElute spin columns sometimes gave rise to weak, unspecific fluorescent signals on the NanoChip array. After PCR amplification and purification of the PCR products, the DNA concentration was determined. Different dilutions with final concentrations of 1, 3, 5, 10 and 20 fmol/µl were made in a total volume of 50 µl. From each dilution, 10 µl was addressed to the NanoChip array, 10 µl was used for gel electrophoresis and 2×10 µl were used for DNA quantification of the diluted PCR product. Figure 1 shows the results for TAT and SRY8299. Hybridization of the fluorescently labelled reporters to the PCR products were analyzed at different temperatures. The images shown in Fig. 1 were selected because they best illustrate the differences in sensitivity between the fluorescent signals obtained from PCR products of various lengths. Clear signals were obtained from 10–30 fmol of the 44 bp SRY8299 fragments and the 54 bp TAT fragments. Signals of similar strengths were obtained from 30–50 fmol of the 108 bp TAT fragments and 50–100 fmol of the 167 bp SRY8299 fragments. For fragments longer than 200 bp, more than 200 fmol were needed to give a strong signal at the chosen temperature. At lower temperatures, the signals obtained from the longer PCR products were stronger, but the hybridization was also less stringent and the other fluorescently labelled reporter, designed to detect the other SNP allele, bound stronger to the same position resulting in a lower signal-to-noise ratio. Next, we wanted to analyze how the lengths of the PCR products would influence SNP typing of individual amplicons in multiplexes. We developed three different 4-plex PCRs from the singleplex PCRs already tested on the electronic microarray. Each 4-plex contained one amplicon for each of the four Y chromosome SNPs. The short 4-plex included amplicons ranging from 44 to 63 bp. The medium 4-plex included amplicons ranging from 108 bp to 210 bp, and the long 4-plex included amplicons ranging from 165 bp to 316 bp. After loading the 4-plexes on the NanoChip array, it was immediately clear that high concentrations of the long 4-plex were needed to obtain results. Only the shortest fragment in the long 4-plex, the 165 bp SRY1532 fragment, gave a strong signal, whereas the signals from the longer fragments were barely visible even at low temperatures (data not shown). In contrast, the signals obtained from the short and the medium 4-plexes were easily detected, therefore we decided to use the short and the medium 4-plex for further studies. After PCR amplification and purification of the PCR products, the DNA concentration was determined. Dilutions were made as before and the concentration of each dilution was determined. A total of 10, 30, 50, 100 and 200 fmol of each 4-plex were loaded in polyacrylamide gels and in individual pads on the NanoChip array. The images shown in Fig. 2 were selected because they gave a clear indication of the differ-

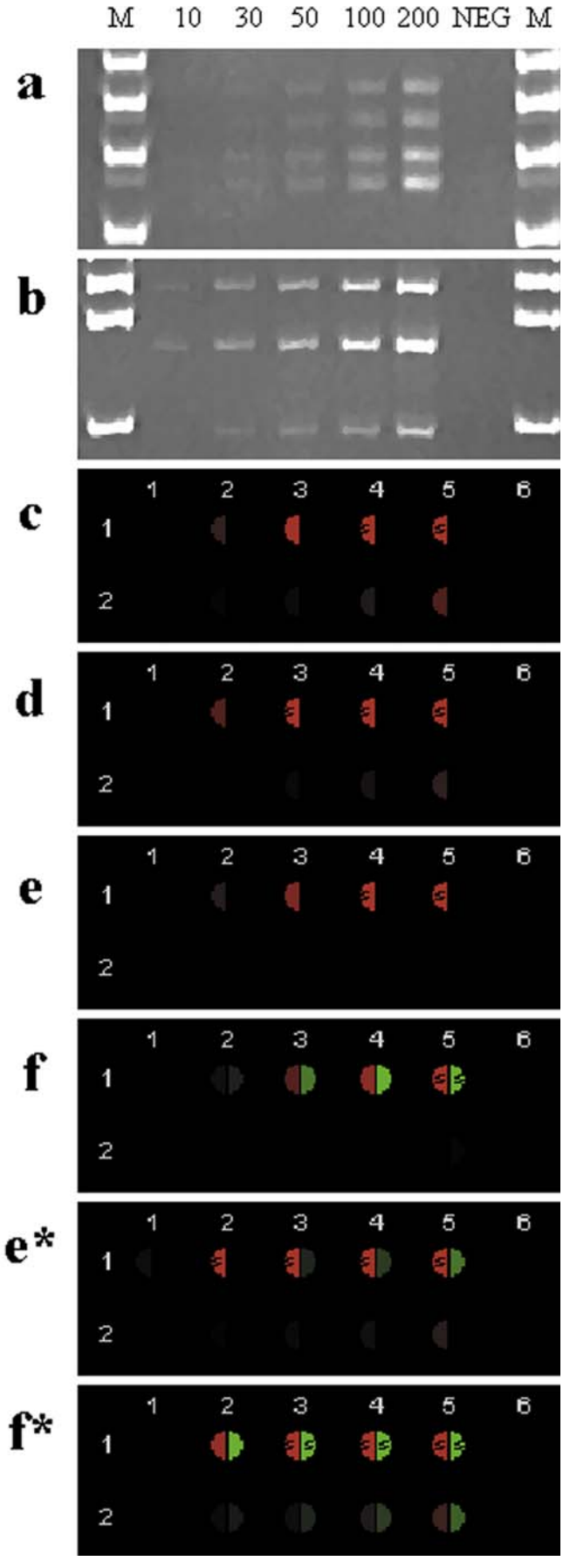

Fig. 2a–f Hybridization experiment with the short 4-plex and the medium 4-plex. A total of 10 µl of 1, 3, 5, 10, and 20 fmol/µl dilutions and a negative PCR control were loaded in polyacrylamide gels and addressed to the NanoChip array. The short 4-plex is shown in *row 1* and the medium 4-plex is shown in *row 2* on the NanoChip array. **a** Gel image of the short 4-plex, **b** gel image of the medium 4-plex, the 165 bp SRY1532 fragment and the 167 bp SRY8299 fragment could not be separated in the gel and the fragments appear as one band. **c** Hybridization with TAT reporters at 33°C, **d** hybridization with SRY1532 reporters at 35°C, **e** hybridization with SRY8299 reporters at 46°C and **f** hybridization with 92R7 reporters at 41°C. **e*** The same hybridization as in (**e**), but at a lower temperature (39°C). **f*** The same hybridization as in **f**, but at a lower temperature (37°C). *s* inside the coloured half circle indicates that the fluorescent signal was saturated

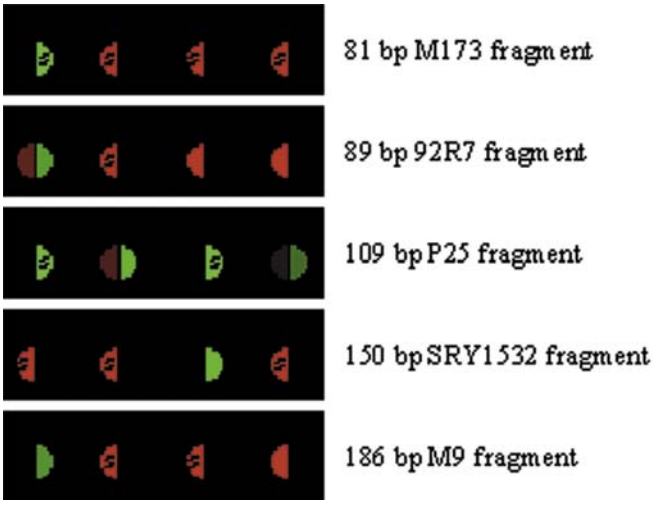

Fig. 3 Examples of a hybridization experiments for the Y chromosome loci SRY1532, M9, M173, P25 and 92R7. Hybridization was performed at 42°C, 39°C, 39°C, 35°C, and 38°C for M173, 92R7, P25, SRY1532, and M9, respectively. *s* inside the coloured half circle indicates that the fluorescent signal was saturated

ences in signal strength between the short and the medium 4-plexes. The signals from the SRY8299 and the 92R7 fragments in the medium 4-plex were barely visible at the selected temperatures (Fig. 2e, f) but at lower temperatures, the SNP allele could be easily determined (Fig. 2e*, f*). At these temperatures, the signals from the SRY8299 and the 92R7 fragments in the short 4-plex were saturated and consequently the correct SNP alleles could not be determined. In conclusion, 30–50 fmol of the short 4-plex was needed to obtain a strong signal for all four fragments, whereas more than 200 fmol of the medium 4-plex was needed to obtain a signal of similar strength. During this work, we learned that the Y chromosome loci 92R7 and P25 (used in the study below) are repeated several times on the Y chromosome and that at least one of the duplicated regions in each group of loci is polymorphic [18]. This explains why we detected two 92R7 and P25 alleles in some individuals (Figs. 2 and 3).

Short amplicons are a contamination risk

Our laboratory is routinely doing PCR-based investigations in crime casework and the laboratory is accredited according to the ISO17025 laboratory standard. We set up PCRs in laminar air flow (LAF) benches placed in a clean room with positive air pressure, and we perform PCR and post-PCR manipulations in separate rooms with negative air pressure. LAF-benches and other work spaces are cleaned with a 10% bleach solution before and after each experiment, and UV light is turned on in LAF-benches between experiments. Furthermore, co-workers who have been inside a post-PCR room are not allowed to enter a clean pre-PCR room later that day. This is done in order to reduce the risk of contamination by PCR products. Nevertheless, we observed contamination in the negative control (no target DNA) or the female control (no Y chromosome target) in 60% of the 52 experiments involving either the short 4-plex or singleplex amplification of the 44 bp SRY8299 fragment, the 54 bp TAT fragment or the 58 bp 92R7 fragment. In contrast, in none of the 7 singleplex experiments involving the 63 bp SRY1532 fragment did we observe contamination of the negative or the female control. Furthermore, we have never observed contamination in experiments involving the longer singleplexes, the medium 4-plex or the long 4-plex, and we routinely work with amplicons in the 70–80 basepair range without having problems with contamination. This indicates that the risk of contamination increases when the amplicons are shorter than 60 bp.

When analyzed by gel electrophoresis, the amounts of the three fragments that sometimes appeared in the negative and the female controls varied, but often they were similar to the amounts amplified from the samples (data not shown) suggesting that a reduction in the number of PCR cycles would not solve the problem. Most of the PCR amplifications were performed by a female technician, thus, reducing the risk of contamination by a member of the staff. The source of the contamination could not be identified from analyses of the three fragments and it was impossible to determine if the contamination originated from more than one source, because TAT and SRY8299 are not polymorphic in the tested population (only Danes were tested) and because 92R7 is duplicated.

Typing of 400 individuals on the Nanogen Molecular Biology Workstation

We have developed a 25-plex PCR containing 35 known Y chromosome SNPs and we have developed a method for detection of all 35 SNPs in one minisequencing reaction [16]. The sizes of the PCR products in the multiplex ranged from 79 to 186 basepairs. We selected five of the SNPs, SRY1532, M9, M173, 92R7 and P25, for analysis on the Nanogen Molecular Biology Workstation. After PCR amplification, the 25 PCR products were purified with the MinElute PCR purification kit. Subsequently, 0.2 µl was used for a minisequencing reaction and 7–10 µl was loaded on the NanoChip array. Only the five selected amplicons were biotin-labelled and, thus, only the five selected amplicons were bound to the NanoChip array. The other amplicons in the 25-plex were washed away before hybridization. Figure 3 shows typical results from four samples. A total of 400 males of varying ethnic origin were typed in duplicate. In all samples, we obtained concording results from the hybridizations performed on the NanoChip array and from the minisequencing reactions analyzed by capillary electrophoresis [19, 20]. Of the samples 2–3% had to be typed again due to weak signals, which were usually caused by low amounts of PCR products. However, in most cases, three or four SNPs could be typed from the sample despite the weak PCR. Pads with no signal at all were observed (<0.5%), but it is unknown whether this was caused by instrumental or manual errors. The signal-to-noise ratios were higher than 10:1 for the vast majority of the samples but a signal to noise ratio of 3:1 was considered acceptable for certain samples. However, this will not be acceptable for analyses of autosomal SNPs where heterozygotes are expected. For autosomal SNPs, an analysis with a signal-to-noise ratio of less than 5:1 must be repeated.

In this study, we used 40–50 bp synthetic oligonucleotide controls to normalize the fluorescent signal. This strategy was chosen because heterozygotes for Y chromosome SNPs do not exist. The strength of the signals obtained from the synthetic oligonucleotides was comparable to those obtained from the short amplicons. However, the synthetic oligonucleotide controls were not the best possible controls, because the secondary structures of the longer amplicons were probably different, and therefore the hybridization efficiencies towards the controls and the longer amplicons were different. For future Y chromosome studies, we recommend the use of mixed samples of DNA from males with different SNP alleles as controls.

Discussion

Today, almost all DNA analyses performed in forensic laboratories are based on PCR of short tandem repeats (STRs) [21]. The STRs are highly polymorphic and sufficient discrimination can, in most cases, be acquired by analyzing 10–15 STRs. Amplification and analysis of STRs has been carefully optimized and with the current technology, up to 16 STRs can be amplified in a single multiplex PCR and analyzed in a single test. One of the disadvantages of STR loci are the relatively long PCR products. The STR amplicons amplified by the commercially available STR kits are 100–400 bp in size. In contrast, SNP loci can be amplified by the shortest possible PCR (<50 bp) and therefore SNP loci might be the only markers that can be amplified from highly degraded DNA isolated from e.g. biological samples collected from excavation sites or DNA isolated from crime or disaster scenes. SNPs are usually biallelic and therefore 50–100 SNPs are needed to reach the same level of discrimination as that acquired from 10 STRs [22]. The use of SNPs for human identifi-

cation in forensic investigations will therefore require extensive multiplexing and highly efficient SNP typing technologies.

Here, we show that hybridization of fluorescently labelled reporters to 50 bp PCR fragments immobilized on the NanoChip array is a highly sensitive method for SNP typing. Only 10–30 fmol are needed to obtain a strong signal. We also show that longer amplicons containing the same SNP and hybridized to the same reporters in the same reaction give weaker signals. If the PCR products are 200 bp or longer, more than 200 fmol is needed to obtain a signal similar in strength to the signal obtained from 10–30 fmol of 50 bp amplicons. The size of the PCR products seems even more important for the hybridization to individual amplicons in multiplexes. The three longest amplicons in the long 4-plex, ranging from 267 bp to 316 bp, were difficult to detect even though the same singleplex PCR products were easily detected. The sensitivity of the hybridization was also decreased for the individual amplicons in the medium 4-plex and to a lesser degree for the amplicons in the short 4-plex.

It is well known that the efficiency of minisequencing reactions [23, 24, 25] and the efficiency of antisense inhibition of gene expression [26, 27, 28] strongly depends on the structural context of the target sequence because the minisequencing primers and the antisense probes hybridize inefficiently to target sequences that are embedded in intramolecular or intermolecular structures. The hybridization on the NanoChip array is performed at room temperature in a buffer with a high salt concentration and therefore secondary structures in the DNA target will form rapidly. Compared to long amplicons, short amplicons are less likely to form interactions that will prevent hybridization of the reporters to their target sequence. This is the most likely explanation for the stronger signals that we observed with the short amplicons. Similarly, the decreased sensitivity that was observed in multiplexes may be explained by intermolecular interactions between different amplicons. Alternatively, longer amplicons may move less efficiently in the permeation layer than shorter, more mobile amplicons and consequently, the number of accessible streptavidin molecules in the layer may be smaller for longer amplicons. This will result in a weaker hybridization signal, especially when longer amplicons compete with other amplicons in a multiplex for the same sites. We have tried to increase the electronic addressing time to eliminate this possibility and we found that addressing times longer than 3 min did not increase the sensitivity (data not shown).

Multiplexing is essential for forensic DNA analyses because as much information as possible needs to be extracted from often very little sample material. Therefore, the decreased sensitivity we observed in multiplexes is a matter of concern. In this work, we demonstrate that it is possible to type 5 SNPs on 5 different amplicons in 400 different males with 100% accuracy by the NanoChip SNP typing protocol. The fragments could be amplified from as little as 100 pg chromosomal DNA (data not shown), which is roughly the amount found in 30 cells. The current NanoChip SNP typing protocol is relatively time-consuming because each SNP has to be analyzed one at the time and because stripping, washing and re-hybridization have to be done manually. Obviously, these processes can be automated relatively easily and hopefully, the next generation of the NanoChip platform will have an integrated loader and reader protocol. In our hands, 10–15 rounds of stripping and re-hybridization can be performed on the NanoChip array without decreasing the signal strength (data not shown). In comparison, we can type 35 SNPs in one reaction by minisequencing [16]. This degree of multiplexing does not seem possible with the current NanoChip SNP typing protocol and therefore a new strategy for SNP typing on the NanoChip array is needed for large multiplexes. One possible strategy would be to split up the multiplex on the NanoChip by hybridization of individual amplicons in the multiplex to amplicon-specific captures in different pads. All amplicons could then be analyzed at the same time and the time of analysis would be significantly shortened.

We show here that PCR amplification of short amplicons and hybridization to short amplicons are highly efficient, however, amplicons shorter than 60 bp also seems to pose a contamination risk. In our hands, 60% of the negative controls from experiments involving amplicons shorter than 60 bp were contaminated with short amplicons. In contrast, we routinely work with amplicons in the 70–80 bp range without having problems with contamination. This interesting observation will be further investigated in collaboration with other forensic laboratories.

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